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Analytical Methods

A new acetonitrile-free mobile phase for HPLC-DAD determination of individual anthocyanins in blackcurrant and strawberry fruits: A comparison and validation study

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ABSTRACT

Research focused on identification and quantification of anthocyanins from berries and other fruits is gaining importance due to the observed inverse relationship between anthocyanin intake and the incidence of certain diseases. Separation and quantification of these compounds is mainly achieved on reverse phase HPLC coupled to different detection systems using mostly acetonitrile as the mobile phase of choice. Nevertheless, the scientific community recently faced a worldwide shortage of this solvent which resulted in prices soaring dramatically. In this context, the present study describes the comparison and validation of a newly developed methanol-based method for the identification and quantification of major berry anthocyanins using standard HPLC coupled to photo diode array detection. Moreover, two different commercially available stationary phases were tested. The methanol-based method developed herein showed high repeatability (R.S.D <1.3%), rapidity (<35 min) and accuracy and therefore may be suitable for routine quantification of berry anthocyanins. Comparison with an earlier established acetonitrile-based method showed that despite differences in absolute concentrations between both methods the determined anthocyanin concentrations were highly correlated ($r > 0.95$). Method validation was further achieved by elucidating differences in the anthocyanin profile between different blackcurrant and strawberry cultivars.

1. Introduction

Quality of berries, including strawberry and blackcurrant fruits, is partially characterised by traits such as colour and taste. Among the compounds responsible for the characteristic colour of both blackcurrant and strawberry fruits are the so called polyphenolic compounds, specifically anthocyanins; the type of polyphenols which are quantitatively most important (Lopes da Silva, Escribano-Bailon, Perez Alonso, Rivas-Gonzalo, & Santos-Buelga, 2004). Anthocyanins are amongst the most important fruit pigments visible to the human eye, and in the particular case of blackcurrant and strawberry fruits are responsible for their purple and red colour at maturity, respectively (Andersen, 2002). The anthocyanidins are the basic constituent of anthocyanins consisting of an aromatic ring (C_6) bonded to an heterocyclic ring (C_3) containing oxygen and bonded with a third aromatic ring (C_6) (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The differences between anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of these sugars and the nature and number of aliphatic or aromatic acids attached to the sugars (Fig. S1). To date more than 500 anthocyanins made up of 23 different anthocyanidins have been reported. This said, just six are commonly present in fruits and vegetables (FAV) and flowers (*viz.* cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin). Berries, including strawberries and blackcurrants, are undoubtedly one of the richest sources of anthocyanins with reported concentrations up to 180 and 400 $\mu g\ g^{-1}$ FW, respectively (Giné Bordonaba & Terry, 2008; Terry, Chope, & Giné Bordonaba, 2007). Increasing interest in the anthocyanin concentration of several FAV is, however, not only due to their potential as natural colourants but also to their associated health-promoting properties. Despite the enormous variability between individuals, estimated consumption of anthocyanins in the United States is in the order of 12.5 mg day^{-1} per person (McGhie & Walton, 2007; Wu et al., 2006), whereas consumption in Europe has been estimated as *ca.* 20 mg day^{-1} per person (Andersen, 2002; de Pascual-Teresa & Sánchez-Ballesta, 2008) or even greater in certain European countries such as Finland with values of 82 mg day^{-1} per person (McGhie & Walton, 2007; Wu et al., 2006). Nowadays, and regardless of the poor bioavailability of these compounds (Nielsen,

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Dragsted, Ravn-haren, Freese, & Rasmussen, 2003) increasing evidence suggest that anthocyanins, as natural antioxidants, exert anticarcinogenic, antiinflamatory, vasoprotective and anti-obesity effects when tested *in vitro* or *in vivo* (Prior, 2004; Schantz, Mohn, Baum, & Richling, 2010).

As a result of the suggested health-related properties of anthocyanins, the number of studies trying to extract, identify and quantify these compounds from different food sources has steadily increased in recent years. Generally, these polar pigments are extracted using aqueous mixtures of either ethanol, methanol or acetone (Kahkonen, Hopia, & Heinonen, 2001); this said, discrepancies exist in whether acetone or methanol based solvents are more efficient for the extraction of these compounds from different FAV (Giné Bordonaba & Terry, 2008). Separation and quantification of anthocyanins is generally achieved on reversed phase HPLC coupled to different detection systems (Lee, Rennaker & Wrolstad, 2008) (*viz.* Photo diode array (PDA), mass spectrometry (MS)) and use mostly acetonitrile as the mobile phase of choice due to its elution strength, low viscosity, and good miscibility with water (Table 1). Nevertheless, the scientific community recently faced a worldwide shortage of this solvent due, in part, to the global economic downturn and consequently the recent near collapse of the automotive industry from which acetonitrile is obtained as a by-product in the production of acrylonitrile. Restrictions over the use of acetonitrile and consequent dramatic price increases resulted in standard elution of anthocyanins being problematic as no viable replacement was really available.

In this context, the aim of the present study was to clarify, using a simple single-step extraction procedure, whether methanol- or acetone-based solvents were more efficient for extracting anthocyanins from strawberry fruits, and further to study the potential of a methanol-based mobile phase that could readily replace the widely used acetonitrile for the chromatographic separation and identification of main anthocyanins in both blackcurrant and strawberry fruits. Hence, different HPLC operating conditions including two different columns and two different mobile phases were tested. The anthocyanin content of different strawberry and blackcurrant cultivars was examined with the most suitable method.

2. Material and methods

2.1. Plant materials

Strawberry fruits from three different cultivars (*viz.* Antea, Clery and Matis) with known differences in their anthocyanin profile (Crespo, Giné Bordonaba, Terry & Carlen, 2010) were used for method comparison and validation with a previous method carried out on strawberry fruits (Terry et al., 2007). Fruits were grown open-fields in, Switzerland (46°12′ N, 7°18′ E) using conventional agronomic practises (Crespo et al., 2010). Blackcurrant berries from cultivars Ben Dorain, Ben Gairn and Ben Tirran were supplied by GlaxoSmithKline Plc. Bushes were grown in open-field at Norfolk, UK (52°39′ N, 0° 54′ E) under standard commercial practises (Rob A. Saunders, *pers. comm.*). All plant materials were harvested at commercial fully ripe stage. Half cut strawberries or full blackcurrant berries were snap-frozen in liquid nitrogen and stored briefly at −40 °C before being freeze-dried in an Edwards Modulyo freeze drier (W. Sussex, UK) for 5 days at 0.015 kPa. Lyophilised samples (Giné Bordonaba & Terry, 2009) were then ground in a pestle and mortar, weighed and returned to the freezer until further analysis.

2.2. Anthocyanin extraction

Individual anthocyanins were extracted as described elsewhere (Giné Bordonaba & Terry, 2008; Terry et al., 2007) by mixing 150 mg of freeze-dried sample with 3 ml of 70% (v/v) methanol and 0.5% (v/v) HCl in HPLC-grade water. The choice of extraction solvent was based on previously published work (Giné Bordonaba & Terry, 2008) and was compared to an acidified acetone aqueous extraction solvent (70% acetone (v/v) and 0.5% (v/v) HCl in HPLC-grade water) (Anttonen & Karjalainen, 2006; Awika, Rooney, and Waniska (2005)) using strawberries and blackcurrant berries as model fruits. The slurry obtained was held, inside clear 5 mL vials, at 35 °C in a closed water bath with constant shaking for 1.5 h; mixing the samples every 15 min. Finally, the flocculate obtained was filtered through a 0.2 μm Millex-GV syringe driven filter unit (Millipore Corporation, MA) and the clear extract analysed by HPLC.

2.3. Extraction recovery

The recovery values for the different anthocyanins naturally occurring in several berries (*viz.* delphinidin 3-O-glucoside (delp-3-glu), cyanidin 3-O-glucoside (cya-3-gluc), cyanidin 3-O-rutinoside (cya-3-rut), pelargonidin-3-glucoside (pg-3-glu), malvidin-3-glucoside (malv-3-gluc); Extrasynthèse, Lyon, France) were investigated. Samples used for extraction recovery studies corresponded to either strawberry or blackcurrant pooled samples from different cultivars. Briefly, freeze-dried extracts were prepared in triplicate, as described previously, and the anthocyanin content determined from the calibration curve obtained with external standards. The same freeze-dried sample (in triplicate) was then spiked (standard additions) with known concentrations (0.5 mg g^{-1} DW) for each standard (delp-3-gluc, cya-3-gluc, cya-3-rut, pg-3-gluc and malv-3-gluc) and extracted following the same procedure as described earlier. The recovery (%) was calculated as the ratio [(anthocyanin concentration in the spiked extract − anthocyanin concentration naturally present in the extract)/(spiked anthocyanin concentration)].

2.4. HPLC measurements

The anthocyanin profile of blackcurrant and strawberry fruits was determined using an Agilent 1200 series HPLC binary pump system (Agilent, Berks., UK) equipped with a Agilent 1200s DA G1315B/G1365B photodiode array with multiple wavelength detector. Strawberry diluted (1:5 v:v) extracts were injected (10 μL) into either a Zorbax Eclipse XDB-C18 column of 250 mm × 4.6 mm diameter, 5 μm particle size with an XDB-C18 guard column of 12.5 mm × 4.6 mm diameter (Stationary Phase (SP) 1) or an Alltech Allsphere ODS-1 column of 250 mm × 4.6 mm diameter, 5 μm particle size (Alltech; Part No. 778357) with an Alltech Allsphere ODS-1 guard column of 7.5 mm × 4.6 mm diameter (Part No. 96402) (SP 2) (Giné Bordonaba & Terry, 2008; Terry et al., 2007). A detailed study on the performance and characteristics of the stationary phases described herein was done earlier (Gilroy, Dolan, Carr, & Snyder, 2004). The different mobile phases (MP) tested consisted of 2% (v/v) acetic acid in HPLC-grade water (A) and 2% (v/v) trifluoroacetic in methanol (B) (MP1) or that previously described by Terry et al. (2007) (MP2); 1% (v/v) phosphoric acid (Acros Organics, Leics., UK) and 10% (v/v) acetic acid (Fischer Scientific, Leics., UK) in HPLC-grade water (A) and acetonitrile (B). The gradient conditions for MP1 was 0–10 min, 2–20% B; 10–20 min, 20–25% B; 20–25 min, 25–35% B; 25–35 min, 35–75% B. Flow rate was, in both cases, set up at 1 mL min^{-1} and the column temperature set at either 35 or 40 °C using an Agilent G1316A thermostated column compartment for SP1 and SP2, respectively. Prior to be injected into the HPLC systems, sample in dark vials were kept at 4 °C using an Agilent G1330B cooled autosampler.

After comparison of the different HPLC operating conditions, validation studies were performed by means of the optimum